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Clinical compatibility Between Negative Stains, Quick Gram Chromotrope, Gram And Giemsa Staining Techniques For Detection Of *C.Parvum* Infection In Children Under Five Years

Ali Ibrahim Ali AL-Ezzy*, Abeer Thair Khadim
Department of pathology, College of Veterinary Medicine, University of Diyala, Iraq.
Department of Medicine, College of Veterinary Medicine, University of Diyala, Iraq.

Corresponding author :alizziibrahim@gmail.com
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Abstract :

Aim : To investigate the agreements between chromotrope gram hot, gram, Giemsa , nigrosin and malachite green staining techniques for detection of *C.Parvum* infection in children under 5 years in Baqubah-Diyala province

Methods : One hundred diarrheic children under 5 years were included .Stool samples were examined after stained with hot Ziehl –Neelsen , Chromotrope Gram Hot; Gram ,Giemsa, Nigrosin and malachite Green.

Results :

ZN hot and all alternative staining techniques were identical in diagnosis of (26%) negative *C.Parvum* infection among diarrhea cases in children .A total of (9%) with low score in chromotrope Gram Hot ,(10%) moderate and (19%) have heavy score in both techniques. Moderate agreement (kappa =0.514),was reported between ZN hot and chromotrope Gram Hot for diagnosis of *C.Parvum* oocysts in children . A total of (11%) of cases have low score in ZN hot and Gram Hot staining , (8%) were moderate and (19%) have heavy score in both techniques. Moderate agreement (kappa =0.525),was reported between ZN hot staining and Gram Hot . A total of (12%) of cases have low score in ZN hot staining and Giemsa; (10%)were moderate and (18%) have heavy score in both techniques. Moderate agreement (kappa =0.552),was reported between ZN hot staining and Giemsa for diagnosis of *C.Parvum* oocysts . A total of (9%) of cases have low score in ZN hot staining and nigrosin, (13%) were moderate and (28%) have heavy score in ZN hot staining and nigrosin. Moderate agreement (kappa =0.665),was reported between ZN hot staining and Nigrosin. A total of (10%) have low score in ZN hot staining and malachite green ,(8%) were moderate and (19%) have heavy score in both techniques .Moderate agreement (kappa =0.515),was reported between ZN hot staining and malachite green.

Conclusion : All Alternative stains for Hot ZN(Chromotrope Gram Hot; Gram ,Giemsa, Nigrosin and malachite Green have identical specificity (100%) for diagnosis of negative *C.Parvum* among diarrhea cases in children . Moderate agreement was reported between ZN hot staining and all alternative stains (Chromotrope Gram Hot ; Gram Hot ,Giemsa , Nigrosin and Malachite Green) for diagnosis of *C.Parvum* oocysts in children which represent good alternatives in rural areas and low income countries

Key words :cryptosporidium parvum ,negative stains, Basic stains , diarrhea ,Iraq

How to cite the article

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Introduction

Cryptosporidium is intracellular protozoan parasite that live in the gastrointestinal tract of mankind and many other vertebrates animals[1]. They are the widespread source of diarrheal disease among both immunocompetent and immunocompromised individuals throughout the world leading to considerable morbidity and mortality, especially in developing countries [2] and in individuals less than five years[3]. Cryptosporidiosis is a diarrheal disease caused by microscopic parasites of the genus Cryptosporidium [4] . The parasite is protected by an outer shell that allows it to survive outside the body for long periods of time and makes it very resistant to chlorine disinfectants. Both the disease and the parasite are commonly known as "Crypto[5]. Most cases of human cryptosporidiosis are due to infections with the human specific C. hominis or the zoonotic C. parvum[6].Other Cryptosporidium species have also been detected in humans, although less frequently [6, 7] . Current evidence indicates that ruminants are a reservoir of zoonotic Cryptosporidium from where humans get infected by contaminated food and water or through direct contact with livestock, for example animal handlers [8, 9].Twenty six Cryptosporidium species and nearly 50 genotypes have been recognized and described and still new genotypes are being discovered [10].At least ten Cryptosporidium species and four genotypes can infect humans. C. hominis and C. parvum are internationally the most commonly species infecting humans.[10, 11] . Humans can acquire cryptosporidium infections through several transmission routes such as person to person transmission, zoonotic transmission, food borne transmission and waterborne transmission [12].A single oocyst is sufficient to cause infection and disease [13]

When excreted, Oocysts are directly infectious and are able to survive for up to 6 months in a moist and cool environment. In water, oocysts remain viable for 140 days [14]. In immunocompetent persons, cryptosporidium infection usually asymptomatic. in children under the age of five and in immunosuppressed people, the infection leads to severe diarrhea. Nausea, vomiting, discomfort and low-grade fever are other clinical symptoms which may occur during an infection with Cryptosporidium [3]. Symptoms in immunocompromised patients can be very severe and even death has been described[15] .In developing countries 45% of the children are experiencing an infection before the age of two[16]

Current study aims to investigate the agreements between chromotrope gram hot, gram, Giemsa , nigrosin and malachite green staining techniques for detection of *C.Parvum* infection in children under 5 years in Baqubah-Diyala province

Material and Methods

Study area and study population

This study was conducted on 100 newborn to less than 5 years old Iraqi children, living in the Baqubah city -Diyala province 33°45'34.71"N; 44°36'23.97"E ,Northeast [17-20]

Stool Samples collection and processing

The stool samples were collected from 100 children less than 5 years of age suffering from gastrointestinal illness. Sample collection took place from November 2016 to June 2017. The inclusion criterion was diarrhoea, defined as passage of three or more loose or liquid stools per day, or more frequently than is normal for the individual [7, 21-23]

The samples were obtained from Albatul teaching hospital-Baqubah . An ethical consideration and consent by the parents or guardians of the children was signed before getting the samples[24] . The samples were

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collected in a special tightly capped leak proof containers. Each sample was labeled with the child's name, gender and age[22]. Each sample was divided into two portions, one used for immediate examination ,other one preserved and stored in 10% formalin. One volume of the fecal sample was mixed thoroughly using wooden applicator stick, with 3 volumes of 10% formalin. The sample was mixed again, and the specimen containers were sealed well. All samples were reinforced with parafilm, the container was inserted in a plastic bag, and samples were stored at 4ºC in the clinical pathology laboratory at college of veterinary medicine ,Diyala university .

Methods:

Staining Techniques

1) Ziehl–Neelsen staining (ZN)

A. Cold method of ZN Staining Of Fecal Smears (Modified Kinyoun's Acid-Fast Stain) This technique was applied according to [9, 25].A total of 200 fields was examined using 40× and 100× to confirm the diagnosis according to oocysts morphology[3].

B. Hot method of ZN staining of fecal smears :Solutions for the hot ZN staining method according to [26, 27] . In the hot method, Thin smears of fecal sediment were made on a clean grease free glass slide and air-dried. Then, the smears were fixed transiently over a flame. The smears were flooded with basic fuchsin-phenol stain[3]. The slide was heated until the steam appeared without boiling at room temperature for 10 minutes[9]. The smears were then washed in running water for 1-2 min. Then, the slides were decolorized in 5% sulfuric acid for 30 second and counterstained with 3% methylene blue for 1 minute and air-dried. A total of 200 fields was examined using 40× and 100× to confirm the diagnosis according to oocysts morphology.

2) Negative staining methods

a. Nigrosin staining: The procedure according to [28] :

Fecal sample spun 800g for 5 minutes. A drop of fecal sample was smeared on a clean microscope slide. A drop of 1% Nigrosin stain were mixed on a glass slide permit to air dried and fixed by one pass over the flame. The sample was examined using X40 and 100 X objectives.

***b.* Malachite green staining*:* Malachite green staining technique was done according to [29] ,with slight modifications.**

a) Fecal sample spun 800g for 5 minutes .A drop of fecal sample sediment was smeared on a clean microscope slide. A drop of 5% malachite green stain were mixed on a glass slide. Permit to air dried and fixed by one pass over the flame.

b) The sample was examined using X40 and 100 X objectives.

3) Quick Hot Gram Chromotrope Staining [30].

Procedure:

a) Smear was fixed with heat (3 times for 1 second each over a low flame . Then Cooled to room temperature.

b) Then Gram's stain was Performed, omitting the safranin step as follows:

i. slides were flooded into Crystal Violet solution and let stand for 30 seconds.

ii. Then rinsed off excess stain gently with water.

iii. Slides were flooded into Gram's iodine solution and allowed to remain on the slide for 30 seconds.

iv. Gram's iodine solution was removed by gently rinsing with decolorizer solution. The slide was holed at an angle and add the decolorizer solution drop wise until it flows off the slide colorless

v. The slide was washed gently with cold water to remove excess decolorizer solution.

c) Perform chromotrope stain as follows:

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a) The slide was placed in warmed (50° to 55°C) chromotrope stain for one minute.
b) Rinsed in 90% acid alcohol for 1 to 3 seconds.
c) Rinsed in 95% ethanol for 30 seconds.
d) Rinsed in 100% ethanol twice for 30 seconds
e) Let dry then mounted with DPX
In fecal samples, spores should appear as dark staining violet ovoid structures against a pale green background .

4) Gram's stain

This staining procedure was according to [31].

a. Smear was prepared and heat gently to fix
b. The slide was flooded with 0.5% methyl/crystal violet and leave for 30 second.
c. The slide the tilted and sufficient amount of (1%) Lugol's iodine was poured to wash away the stain, cover the slid was covered with fresh iodine and allow to act for 30 sec
d. The slide was tilted and the iodine was washed out with 95 - 100% ethanol or acetone until colour ceases to run out of the smear
e. The slid was rinsed with water
f. Then 0.1% counterstain (safranin or carbol fuchsin) was poured on and leave to act for about 2 min
g. The slid then washed with water and blot dry.
h. cryptosporidium appear as a pink red when examined microscopically.

5) Giemsa Stain [31, 32]

A 1 in 10 dilution of Giemsa's stain was freshly prepared in buffered water. A faecal smear was prepared and allowed to air dry, then fixed in methanol for 60 second. Then the methanol was tipped off and the slide was flooded with diluted Giemsa's stain and leaved for 20 - 25 minutes. Then the slide was rinsed with tap water to remove the stain and to prevent precipitation on the smear. then the slide was allowed to air dry.

A. Scoring system and reporting of oocyst results :

Scoring system for positive sample was used, based on the number of oocyst under x40/ x100 objective lens [33]

Low (+): only one oocysts per high power field x40/ x100.

Moderate (++):2-10 oocysts per high power field x40/ x100.

Heavy (+++):11 or more oocysts per high power field x40/ x100

index of agreement. The level of confidence limits was 0.095 and Here is one possible interpretation of Kappa value [3, 9, 21, 37].

Poor agreement = Kappa value Less than 0.20 (b)Fair agreement = Kappa value 0.20 to 0.40

Moderate agreement = Kappa value 0.40 to 0.60 (d) Good agreement = Kappa value 0.60 to 0.80

Very good agreement = Kappa value 0.80 to 1.00

Statistical Analysis

Demography and cross tabulation were calculated by Statistical analysis using SPSS for windows TM version 17.0[34]. Chi square was used to verify possible association between infection and exposure with different factors[35]. Values were considered to be statistically significant when the p-value obtained was less than 0.05[36].The concordance of the Zn hot and other staining techniques was studied using the Cohen's kappa

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Results :

Agreement between Chromotrope Gram Hot Score and ZN hot staining technique for detection of *C.Parvum* infection in children

As shown in Table(1) ,figures(1 &2), both of ZN hot technique and **Chromotrope Gram Hot** staining were identical in diagnosis of negative *C.Parvum* among diarrhea cases in children , (26%).A total of (9%) of cases were recorded as positive with low score in both techniques (10%) were moderate and (19%) were recorded as positive with heavy score in both techniques. A total of (11%) of cases were reported as having low score of *C.Parvum* oocysts using **Chromotrope Gram Hot** and have moderate oocysts score using ZN hot staining procedure. A total of (7%) of cases were reported as having low score of *C.Parvum* oocysts using **Chromotrope Gram Hot** and have heavy oocysts score using ZN hot staining procedure. A total of (2%) of cases were reported as having moderate score of *C.Parvum* oocysts using **Chromotrope Gram Hot** and have low oocysts score using ZN hot staining procedure. A total of (11%) of cases were reported as having moderate score of *C.Parvum* oocysts using **Chromotrope Gram Hot** and have heavy oocysts score using ZN hot staining procedure. A total of (1%) of cases were reported as having heavy score of *C.Parvum* oocysts using **Chromotrope Gram Hot** and have low oocysts score using ZN hot staining procedure. A total of (4%) of cases were reported as having heavy score of *C.Parvum* oocysts using **Chromotrope Gram Hot** and have moderate oocysts score using ZN hot staining procedure. Significant difference as well as correlation regarding the detection and scoring of *C.Parvum* oocysts among children was reported between **Chromotrope Gram Hot** and ZN hot staining procedure (P value=0.000).Moderate agreement (kappa =0.514, p value= 0.000),was reported between ZN hot staining procedure and **Chromotrope Gram Hot** for diagnosis of *C.Parvum* oocysts in children .

Agreement between Gram Score and ZN hot staining technique for detection of *C.Parvum* infection in children

As shown in Table(2) figures(1&3), both of ZN hot technique and **Gram Hot** staining were identical in diagnosis of negative *C.Parvum* among diarrhea cases in children , (26%).A total of (11%) of cases were recorded as positive with low score in both techniques (8%) were moderate and (19%) were recorded as positive with heavy score in both techniques. A total of (12%) of cases were reported as having low score of *C.Parvum* oocysts using **Gram Hot** and have moderate oocysts score using ZN hot staining procedure. A total of (7%) of cases were reported as having low score of *C.Parvum* oocysts using **Gram Hot** and have heavy oocysts score using ZN hot staining procedure. A total of (1%) of cases were reported as having moderate score of *C.Parvum* oocysts using **Gram Hot** and have low oocysts score using ZN hot staining procedure. A total of (11%) of cases were reported as having moderate score of *C.Parvum* oocysts using **Gram Hot** and have heavy oocysts score using ZN hot staining procedure. A total of (5%) of cases were reported as having heavy score of *C.Parvum* oocysts using **Gram Hot** and have moderate oocysts score using ZN hot staining procedure. Significant difference as well as correlation regarding the detection and scoring of *C.Parvum* oocysts among children was reported between **Gram Hot** and ZN hot staining procedure (P value=0.000).Moderate agreement (kappa =0.525, p value= 0.000),was reported between ZN hot procedure and **Gram Hot** for diagnosis of *C.Parvum* oocysts in children

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Table(1): Agreement between Chromotrope Gram Hot Score and ZN hot staining technique for detection of *C.Parvum* infection in children

Chromotrope Gram Hot Score	ZN hot score in children				
	Negative	Low	Moderate	Heavy	Total
Negative	26(26%)	0(0%)	0(0%)	0(0%)	26(26%)
Low	0(0%)	9(9%)	11(11%)	7(7%)	27(27%)
Moderate	0(0%)	2(2%)	10(10%)	11(11%)	23(23%)
Heavy	0(0%)	1(1%)	4(4%)	19(19%)	24 (24%)
Total	26(26%)	12(12%)	25(25%)	37(37%)	100(100%)
$\chi 2$	137.110				
P value	0.000				
R	0.82971				
P value	0.000				
Kappa	0.514				
P value	0.000				

Table(2): Agreement between Gram Score and ZN hot staining technique for detection of *C.Parvum* infection in Children

Gram Score	ZN hot score in children				
	Negative	Low	Moderate	Heavy	Total
Negative	26 (26%)	0 (0%)	0 (0%)	0 (0%)	26 (26%)
Low	0(0%)	11(11%)	12(12%)	7(7%)	27(27%)
Moderate	0(0%)	1(1%)	8(8%)	11(11%)	23(23%)
Heavy	0(0%)	0(0%)	5(5%)	19(19%)	24 (24%)
Total	26(26%)	12(12%)	25(25%)	37(37%)	100(100%)
$\chi 2$	131.6133				
P value	0.000				
R	0.827				
P value	0.000				
Kappa	0.525				
P value	0.000				

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Agreement between Giemsa Score and ZN hot staining technique for detection of *C.Parvum* infection in children :

As shown in Table(3) figures(1&4), both of ZN hot technique and Giemsa staining were identical in diagnosis of negative *C.Parvum* among diarrhea cases in children , (26%).A total of (12%) of cases were recorded as positive with low score in both techniques (10%) were moderate and (18%) were recorded as positive with heavy score in both techniques. A total of (10%) of cases were reported as having low score of *C.Parvum* oocysts using Giemsa and have moderate oocysts score using ZN hot staining procedure. A total of (8%) of cases were reported as having low score of *C.Parvum* oocysts using Giemsa and have heavy oocysts score using ZN hot staining procedure. A total of (11%) of cases were reported as having moderate score of *C.Parvum* oocysts using Giemsa and have heavy oocysts score using ZN hot staining procedure. A total of (5%) of cases were reported as having heavy score of *C.Parvum* oocysts using Giemsa and have moderate oocysts score using ZN hot staining procedure. Significant difference as well as correlation regarding the detection and scoring of *C.Parvum* oocysts among children was reported between Giemsa and ZN hot staining procedure (P value=0.000).Moderate agreement (kappa =0.552 , p value= 0.000),was reported between ZN hot staining procedure and Giemsa for diagnosis of *C.Parvum* oocysts in children .

Agreement between Nigrosin Score and ZN hot staining technique for detection of *C.Parvum* infection in children

As shown in Table(4), figures(1&5), both of ZN hot technique and Nigrosin staining were identical in diagnosis of negative *C.Parvum* among diarrhea cases in children , 26/100,(26%).A total of (9%) of cases were recorded as positive with low score in both techniques (13%) were moderate and (28%) were recorded as positive with heavy score in both techniques. A total of (1%) of cases were reported as having low score of *C.Parvum* oocysts using Nigrosin and have moderate oocysts score using ZN hot staining procedure. A total of (3%) of cases were reported as having low score of *C.Parvum* oocysts using Nigrosin and have heavy oocysts score using ZN hot staining procedure. A total of (2%) of cases were reported as having moderate score of *C.Parvum* oocysts using Nigrosin and have heavy oocysts score using ZN hot staining procedure. A total of (6%) of cases were reported as having moderate score of *C.Parvum* oocysts using Nigrosin and have heavy oocysts score using ZN hot staining procedure. A total of (11%) of cases were reported as having heavy score of *C.Parvum* oocysts using Nigrosin and have moderate oocysts score using ZN hot staining procedure. Significant difference as well as correlation regarding the detection and scoring of *C.Parvum* oocysts among children was reported between **Nigrosin** and ZN hot staining procedure (P value=0.000).Moderate agreement (kappa =0.665 , p value= 0.000),was reported between ZN hot staining procedure and **Nigrosin** for diagnosis of *C.Parvum* oocysts in children

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Table(3): Agreement between Giemsa Score and ZN hot staining technique for detection of *C.Parvum* infection in Children

Giemsa Score	ZN hot score in children				
	Negative	Low	Moderate	Heavy	Total
Negative	26(26%)	0(0%)	0(0%)	0(0%)	26(26%)
Low	0(0%)	12(12%)	10(10%)	8(8%)	30(30%)
Moderate	0(0%)	0(0%)	10(10%)	11(11%)	21(21%)
Heavy	0(0%)	0(0%)	5(5%)	18(18%)	23 (23%)
Total	26(26%)	12(12%)	25(25%)	37(37%)	100(100%)
$\chi 2$	136.14011				
P value	0.000				
R	0.825				
P value	0.000				
Kappa	0.552				
P value	0.000				

Table(4): Agreement between Nigrosin Score and ZN hot staining technique for detection of *C.Parvum* infection in Children

Nigrosin Score	ZN hot score in children				
	Negative	Low	Moderate	Heavy	Total
Negative	26(26%)	0(0%)	0(0%)	0(0%)	26(26%)
Low	0(0%)	9(9%)	1 (1 %)	3(3%)	13(13%)
Moderate	0(0%)	2(2%)	13(13%)	6(6%)	21(21%)
Heavy	0(0%)	1(1%)	11(11%)	28(28%)	40 (40%)
Total	26(26%)	12(12%)	25(25%)	37(37%)	100 (100%)
$\chi 2$	157.7941				
P value	0.000				
R	0.879				
P value	0.000				
Kappa	0.665				
P value	0.000				

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Agreement between Malachite green Score and ZN hot staining technique for detection of *C.Parvum* infection in children

As shown in Table(5), figures(1&6), both of ZN hot technique and **Malachite Green** staining were identical in diagnosis of negative *C.Parvum* among diarrhea cases in children , (26%).A total of (10%) of cases were recorded as positive with low score in both techniques (8%) were moderate and (19%) were recorded as positive with heavy score in both techniques. A total of (1%) of cases were reported as having low score of *C.Parvum* oocysts using **Malachite Green** and have moderate oocysts score using ZN hot staining procedure. A total of (10%) of cases were reported as having low score of *C.Parvum* oocysts using **Malachite Green** and have heavy oocysts score using ZN hot staining procedure. A total of (2%) of cases were reported as having moderate score of *C.Parvum* oocysts using **Malachite Green** and have low oocysts score using ZN hot staining procedure. A total of (8%) of cases were reported as having moderate score of *C.Parvum* oocysts using **Malachite Green** and have heavy oocysts score using ZN hot staining procedure. A total of (4%) of cases were reported as having heavy score of *C.Parvum* oocysts using **Malachite Green** and have moderate oocysts score using ZN hot staining procedure.

Significant difference as well as correlation regarding the detection and scoring of *C.Parvum* oocysts among children was reported between **Malachite Green** and ZN hot staining procedure (P value=0.000).Moderate agreement (kappa =0.515 , p value= 0.000),was reported between ZN hot staining procedure and **Malachite Green** for diagnosis of *C.Parvum* oocysts in children .

Table(5): Agreement between Malachite green Score and ZN hot staining technique for detection of *C.Parvum* infection in children

Malachite Green Score	ZN hot score in children				
	Negative	Low	Moderate	Heavy	Total
Negative	26(26%)	0(0%)	0(0%)	0(0%)	26(26%)
Low	0(0%)	10(10%)	13(13%)	10(10%)	13(13%)
Moderate	0(0%)	2(2 %)	8(8%)	8(8%)	21(21%)
Heavy	0(0%)	0(0%)	4(4%)	19(19%)	40 (40%)
Total	26(26%)	12(12%)	25(25%)	37(37%)	100 (100%)
$\chi 2$	124.8143				
P value	0.000				
R	0.798				
P value	0.000				
Kappa	0.515				
P value	0.000				

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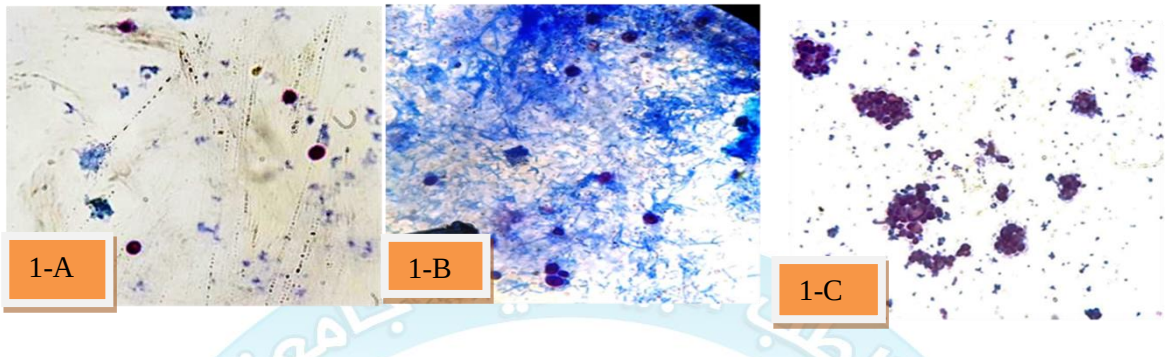

Figure 1. *C.Parvum* oocysts detected in diarrheic stool by ZN hot staining technique In children :A .low score ,B. moderate score, C heavy score (100x)

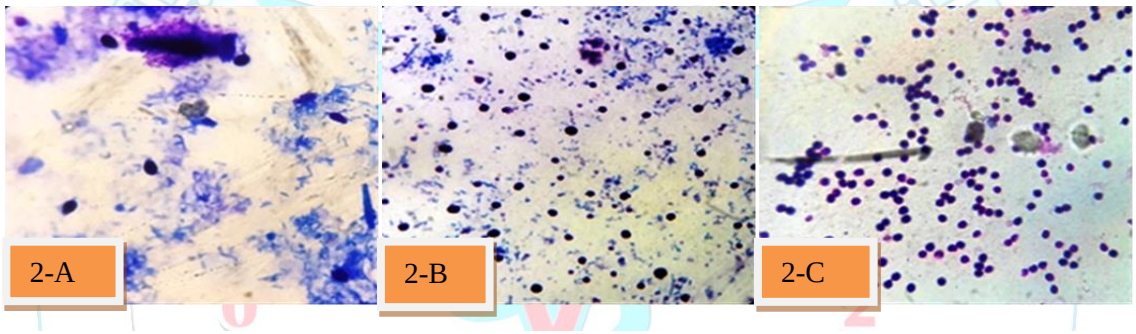

Figure 2. *C.Parvum* oocysts detected in diarrheic stool by Quick Hot Chromotrope –Gram staining technique . In children :A .low score ,B. moderate score, C heavy score (100x).

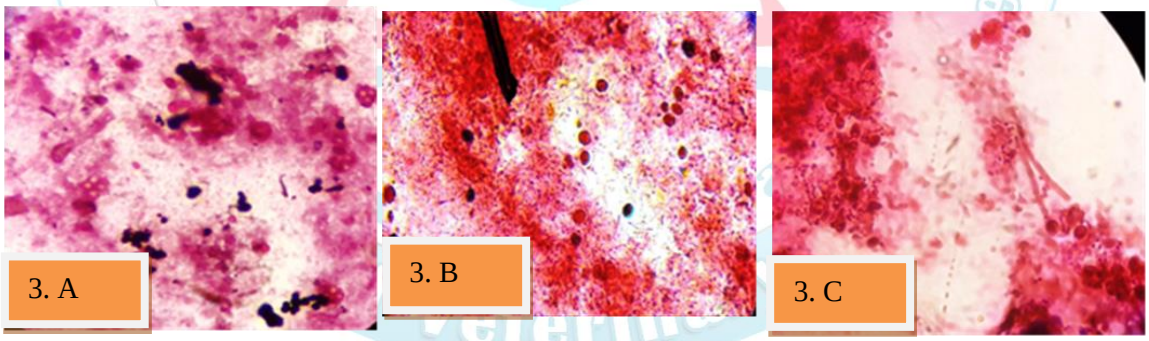

Figure 3. *C.Parvum* oocysts detected in diarrheic stool by Gram staining technique . In children : A .low score ,B. moderate score, C heavy score (100x).)

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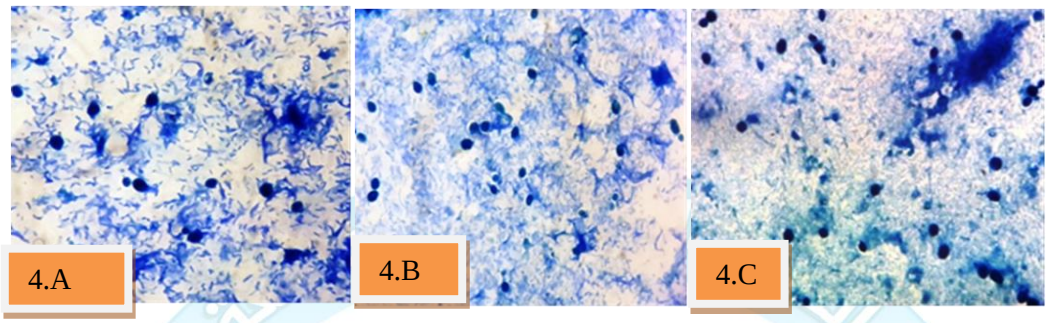

Figure 4 . *C.Parvum* oocysts detected in diarrheic stool by Giemsa staining technique . In children :A .low score ,B. moderate score, C heavy score (100x).

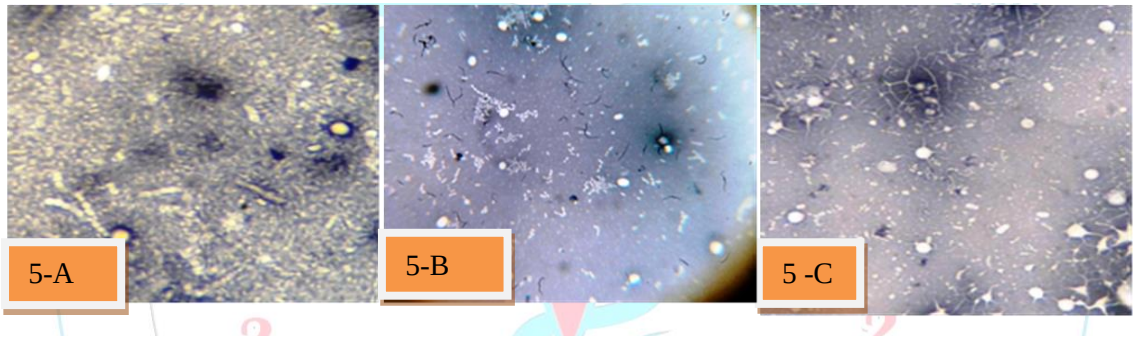

Figure 5. *C.Parvum* oocysts detected in diarrheic stool by Nigrosin staining technique . In children :A .low score ,B. moderate score, C heavy score (100x).

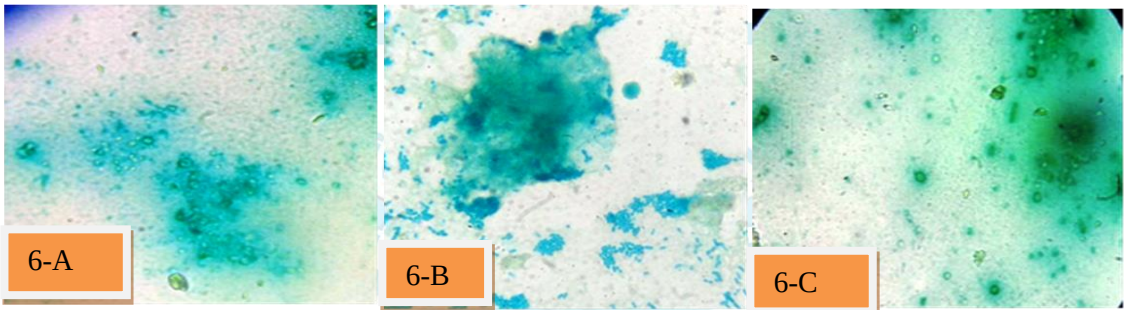

Figure 6. *C.Parvum oocysts detected in diarrheic stool* by Malachite Green staining technique . In children :A .low score ,B. moderate score, C heavy score (100x)

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Discussion

Agreement between Quick hot Chromotrope Gram Score and ZN hot staining technique for detection of *C.Parvum* infection in children

This method is a modification of both chromotrope and Gram's staining techniques and has several advantages like rapid ;inexpensive; and simple differential staining of Microsporidia spores in specimens ; including stool smears[13] . In current study this stain was utilized for detection of cryptosporidium oocysts in fecal samples which appear as deep violet ovoid structures in a relatively clean background after the quick – hot Gram –chromotrope staining technique ,yeast were present in large number in the background, but stained pink-red and were easily differentiated from cryptosporidium oocysts even when present in small numbers[38] . In current study ,significant correlation was reported between Hot Chromotrope Gram and ZN hot staining procedure with moderate agreement (kappa =0.552) in children. This could attributed to the low to moderate number of oocysts excreted from children which reflected to misdiagnosis and scoring

Agreement between Gram Score and ZN hot staining technique for detection of *C.Parvum* infection in children

Significant difference and correlation regarding the detection and scoring of *C.Parvum* oocysts among children was reported between Gram and ZN hot staining procedure (P value=0.000).Moderate agreement (kappa =0.552) in children was reported between ZN hot and Gram staining procedure for diagnosis of *C.Parvum* oocysts.

To overcome disadvantage of gram stain is cryptosporidium appear red in color give the result negative bacteria ,chromorope stain over right this disadvantage as well as the diffrentional bacteria and cryptosporidium in negative stain need experienced eye and well trained laboratory expert , and time consuming . Grams stain appear to be specific and sensitive ,and give good agreement with hot ZN stain

Agreement between Giemsa Score and ZN hot staining technique for detection of *C.Parvum* infection in children

Significant difference and correlation regarding the detection and scoring of *C.Parvum* oocysts among children was reported between Giemsa and ZN hot staining procedure (P value=0.000) which may attributed to interference of oocysts morphology with yeast cells which are pear-shaped and show evidence of budding[39] beside many fecal material that may take-up the bluish discoloration ,making the distinguishing between the cryptosporidium oocysts and others difficult. Moderate agreement (kappa =0.552) in children was reported between ZN hot staining procedure and Giemsa for diagnosis of *C.Parvum* oocysts which come in line with that reported by [40] in India

Agreement between negative stains (Nigrosin & Malachite green) Score and ZN hot staining technique for detection of *C.Parvum* infection in children

The usefulness of nigrosin was to stain the background as well as yeasts and bacteria but not the oocysts, which permit the detection of oocysts accurately but the doubtful cases can be confirmed by the use of ZN staining procedure [41] . Hence moderate agreement between the nigrosin and ZN stains was reported in children for diagnosis of *C.Parvum* oocysts and significant correlation regarding the detection and scoring of *C.Parvum* oocysts among children was reported between nigrosin and ZN hot staining procedure .The difference in scoring

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may attributed to the confusion of oocysts morphology with some fecal materials and even bacterial and fungal spores which can be easily differentiated by experts.

Current results come in contrary with that reported by[42] who consider nigrosin stain is less sensitive than other permanent stains used for detection of *C.Parvum* .The accurate diagnosis of cryptosporidium infections requires fast, cost-effective and sensitive technique [43]

In current study, significant difference and correlation between malachite green and ZN hot staining procedure regarding the detection and scoring of *C.Parvum* oocysts among children and calves was reported. There was moderate agreement between ZN hot procedure and malachite green for diagnosis of *C.Parvum* oocysts in children . This come in line with [41],reported that malachite green staining is sensitive safer for detecting oocysts of cryptosporidium in stool specimens than other staining methods

With the malachite green stain, yeasts were clearly differentiated from oocysts as they took up the stain[41]. Because of the ease with which oocysts can be differentiated from yeasts malachite green negative staining proved to be the most reliable and fastest method of detecting cryptosporidium oocysts, which appeared plump and bright against a dark green background.

Conclusion :

Current study conclude that All Alternative stains for Hot ZN(Chromotrope Gram Hot; Gram ,Giemsa, Nigrosin and malachite Green have identical specificity (100%) for diagnosis of negative *C.Parvum* among diarrhea cases in children , (26%). Moderate agreement was reported between ZN hot staining procedure and all alternative stains (Chromotrope Gram Hot ; Gram Hot ,Giemsa , Nigrosin and Malachite Green) for diagnosis of *C.Parvum* oocysts in children which represent good alternatives in rural areas and low income countries .

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